ABSTRACT

Methicillin Resistant *Staphylococcus aureus* (MRSA) has developed as a prevalent cause of community acquired disease. *S. aureus's* effectiveness is due to its exceptional resistance to host immunity, including nitric oxide (NO•). Full NO• resistance requires activation of a gene subset including *hmp* and *ldh1*. Hmp converts reactive nitric oxide into non-toxic nitrate. Ldh1 relieves redox imbalance incurred during anaerobic respiration required during NO•-stress. Although preliminary work implicated SrrAB for controlling *hmp* expression, the regulation is incomplete, implicating additional regulators. Ldh1 is stimulated by glucose, however the exact regulatory mechanism is unknown; my research goal is to identify the precise regulatory mechanisms for both genes. To find these regulators, I conducted a screen of mutants in every non-essential regulatory *S. aureus* protein by transducing reporter plasmids for each gene into each mutant. Growth curves measuring fluorescence upon NO•-stress to find atypical induction of *hmp* or *ldh1* indicated possible regulators, including SigB and HrcA.

INTRODUCTION

Staphylococcus aureus is a Gram positive, facultative anaerobe that is highly successful human pathogen. As much as 30% of the population carries *S. aureus* on their skin asymptomatically. Methicillin Resistant *Staphylococcus aureus* (MRSA) is an emerging threat that, at one time only present in hospitals, has emerged in the community as a prevalent cause of disease, with approximately 2% of the population carrying MRSA as part of their normal flora. USA 300 is a community-acquired MRSA strain that is currently being studied. The virulence factors that make *S. aureus* such an effective human pathogen limit the efficacy of the innate

immune response by inhibiting opsonization, phagocytosis, and neutrophil recruitment. In addition to these virulence factors, *S. aureus* is also exceptionally resistant to nitric oxide (NO•) (Figure 1).

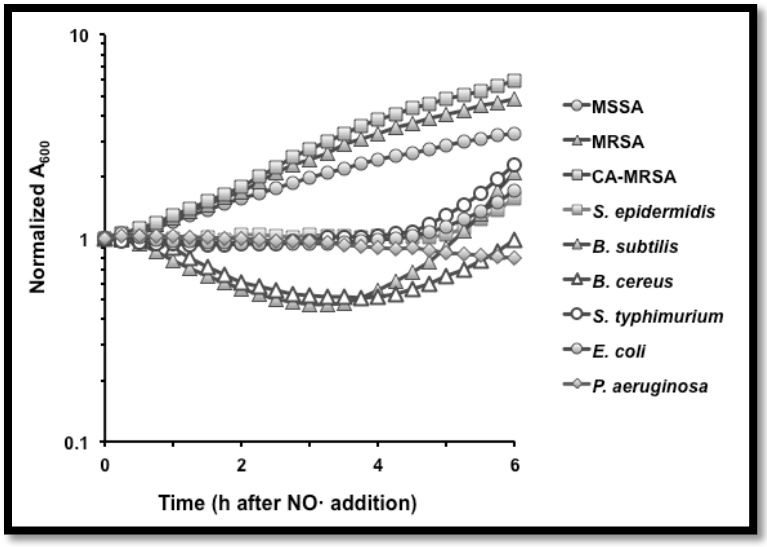

Figure 1. Staphylococcus aureus is uniquely resistant to NO• stress (Richardson lab, unpublished).

The immune system produces NO• to fight off pathogens infecting the body. NO• is a radical that can react with the pathogen's DNA, lipids, and proteins to kill the invading microbes. *S. aureus* accomplishes NO• resistance by activating a subset of genes previously linked to anaerobic metabolism. These genes include *srrAB, hmp,* and *ldh1*. Hmp is an NO• dioxygenase that converts NO• into nitrate. In *Bacillus subtilis*, *hmp* expression has been linked to the SrrAB counterpart, ResDE. SrrAB is a two component system shown to be a global regulator of the shift to anaerobic metabolism in *S. aureus*. In *S. aureus*, however, regulation of *hmp* by SrrAB is incomplete, implicating additional unknown regulators (Richardson, et. al, 2006). Ldh1 is a lactate dehydrogenase which restores redox balance (NADH/NAD+) in the cell. *Ldh1* is repressed by redox sensing regulator Rex, however it is also stimulated by glucose. The mechanism of this glucose related regulation is unknown (Crooke et.al, 2013). This study represents an initial screen of potential candidates for regulators of *hmp* and *ldh1*.

METHODS

NARSA Library

This study utilized a NARSA (Network on Antimicrobial Resistant of *Staphylococcus aureus*) library of 105 mutants in every non-essential regulatory protein in *S. aureus.* The NARSA library was created by the Center for Staphylococcal Research at the University of Nebraska Medical Center through transposon mutagenesis of *S. aureus* strain USA300.

Lysate Preparation

To prepare lysates for transduction of the reporter fusion in to the NARSA library, the strains containing the reporter plasmids with p*rpod::gfp*, p*hmp::gfp* p*ldh1::gfp* (premade) were grown in an overnight culture in BHI and Chloramphenicol. The cultures were then diluted and grown in CY broth for approximately 3 hours, after which, 1 ml of the culture, 1 ml of phage buffer, and 100 µL of a phage stock made from lysate of φ80 RN4220 were combined and allowed to shake at 30 degrees overnight. The lysate was then centrifuged, filter sterilized using .45µM syringe filters, and stored at 4 degrees.

Transduction Protocol

Each regulator mutant in the NARSA library was transduced using the following protocol. The strain was allowed to grow overnight in BHI. In 4 plastic falcons, 1 mL of the overnight culture was added, in addition to 12 µL of 1M CaCl2. To each falcon, lysates from p*rpod*::GFP, p*hmp*::GFP, or p*ldh1*::GFP were added individually, or no lysate was added. The tubes were allowed to incubate for 20 minutes. After incubation, 2 mL of 1% sodium citrate was added to each tube, and each tube was centrifuged for 10 minutes at 4150 rpm. The supernatant

was discarded and resuspended in 2 mLs of TSB + 0.5% sodium citrate. The bacteria were then allowed to rest in a 37 degree water bath for 1 hour. The tubes were then centrifuged again for 10 minutes at 4150 rpm. All but approximately 200 µL of the supernatant was removed; the culture was then resuspended and plated on TSB plates containing 0.5% Sodium Citrate and Chloramphenicol to grow overnight.

Miniprep, PCR, and DNA Gel Electrophoresis Protocol

Two colonies from the transduction plates were selected and grown overnight in BHI and Chloramphenicol. Because of the possibility of spontaneous mutations causing chloramphenicol resistance, each culture must be tested for the presence of the plasmid. This was completed using a QIAprep Spin Miniprep kit. For each culture, 3 mL of culture was pelleted and resuspended in 250 µL of buffer P1 and 12 µL of 2.5 mg/ml lysostaphin in order to break the cell wall of the *S. aureus*. The bacteria were allowed to incubate for 1 hour in a 37 degree water bath. Following, 250 µL of buffer P2 was added to each tube and mixed, proceeded by immediately adding 350 µL of buffer N3. Mixtures were then centrifuged for 10 minutes in a tabletop microcentrifuge. The supernatant was then transferred to a QIA spin prep column and washed with 750 µL of Buffer PE and centrifuged. The QIAprep column was then transferred to a clean microcentrifuge tube and 30 µL of water was added to elute the DNA.

The DNA could then be directly run on a 1% agarose gel to detect the presence of the plasmid. For further verification, PCR using the respective primers could be run to amplify the Plasmid DNA pieces of the Rpod, Hmp, or Ldh1 gene promoters. This verification was only used during the initial stages of the experiment to verify that the strains previously made did not

contain the correct plasmid. Once the strain was verified as containing the correct plasmid, a freezer stock could be made in BHI + 25% glycerol.

Growth Curve Protocol

In order to further identify possible regulatory candidates, growth curves were conducted. The strain to be tested was grown overnight in BHI and Chloramphenicol. 500 µl of the culture was washed twice and resuspended with PBS. Each washed culture was then diluted to an OD660 of 0.01 in PN media with Glucose and Chloramphenicol. The diluted cultures were then aliquoted into a 96 well plate and run on a Tecan Infinite M200 plate reader that measures both OD and fluorescence of each well. At an OD660 of 0.15, 2mM of Nitric Oxide in the form of DETANO was applied to the cells. The Tecan obtained readings for 24 hours.

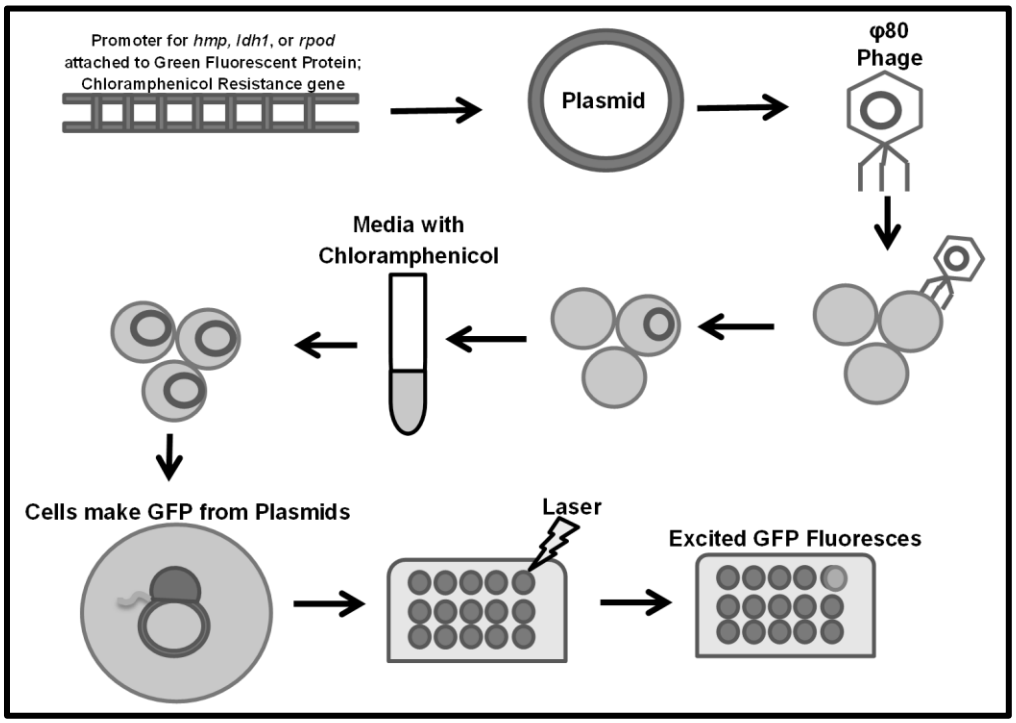

Figure 2. A reporter plasmid containing p*rpod*::GFP, p*hmp*::GFP, or p*ldh1*::GFP and chloramphenicol resistance was inserted into strains of the NARSA library. Bacteria containing the plasmid was then selected for by plating on media containing chloramphenicol. Cellular machinery would then express GFP from the plasmid. When exposed to a laser, this GFP fluoresces in proportion to the amount present. Transcription levels of the gene normally paired with the promoter on the genome can thus be inferred from the fluorescence.

RESULTS

The peak fluorescence of each mutant was averaged and normalized to Optical Density, and to normalized expression of the Wildtype. Significant difference from the Wildtype (values significantly >1 or <1) indicates possible regulators for *hmp* or *ldh1*.

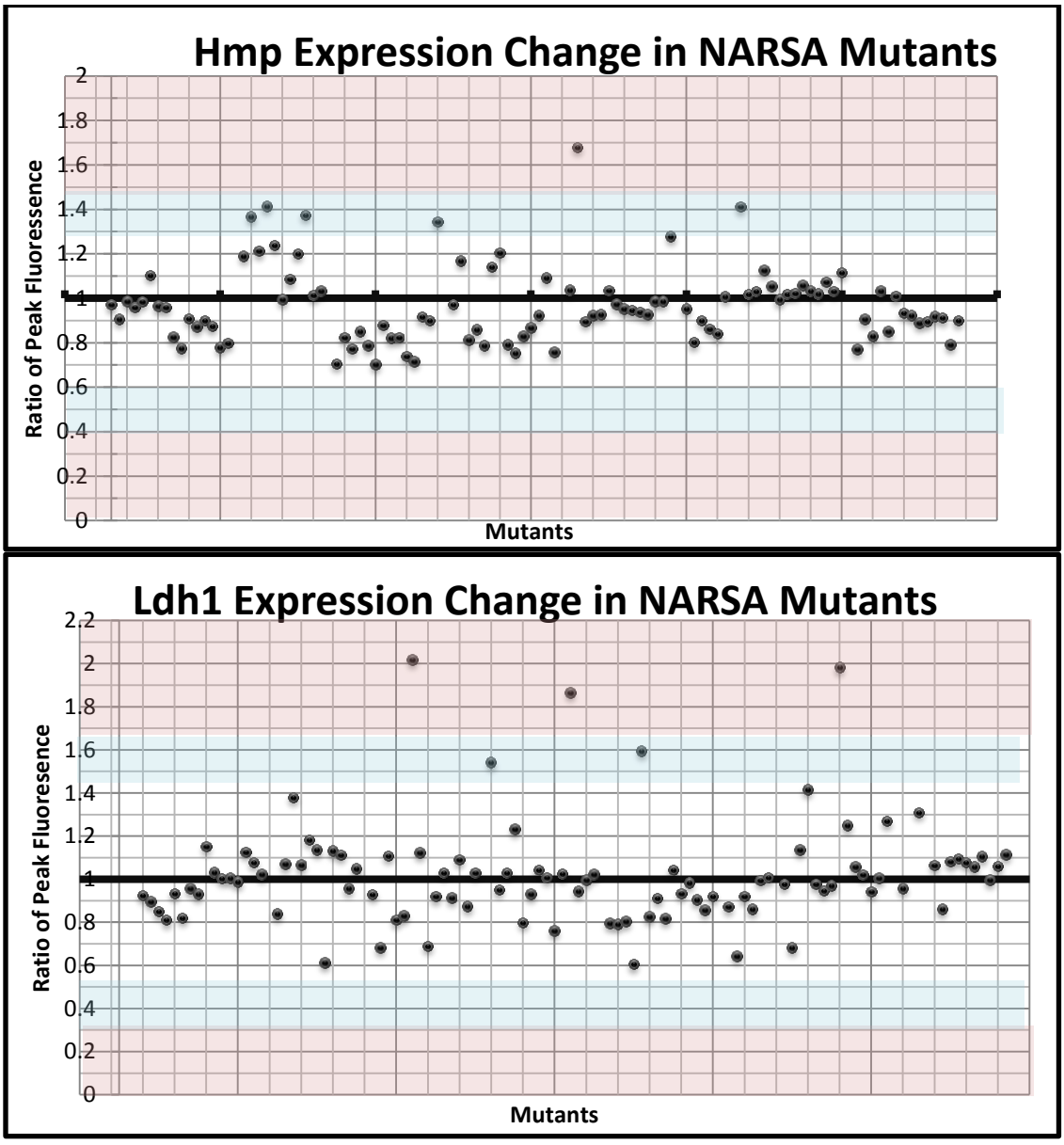

Figure 3. A. (Fluorescence^{mutant} /OD^{mutant}) / (Fluorescence^{WT}/OD^{WT}) ratio of peak fluorescence for mutants containing p*hmp*::GFP compared to the wildtype containing p*hmp*::GFP.
B. (Fluorescence^{mutant} /OD^{mutant}) / (Fluorescence^{WT}/OD^{WT}) ratio of peak fluorescence for mutants containing p*ldh1*::GFP compared to the wildtype containing p*ldh1*::GFP. Δ*rex* (Ratio 34.9) and Δ*srrA* (ratio 6.3) not shown. Points in blue are 2 standard deviations from the average, points in red are 3 standard deviations from the average

Table 1. Mutants that displayed altered hmp expression levels derived from Figure 3A.

NARSA mutant	Ratio of Wildtype	Role of Regulator	
1532	1.277	(agrA) accessory gene regulator protein	
755	1.34	(yfmC) transcription regulator, GntR family	2 STD DEV
367	1.367	(agrR) arginine repressor	
454	1.37	(ArcA) transcriptional regulator, Crp/Fnr family	
415	1.41	putative transcriptional regulator	
1555	1.411	(codY) transcription repressor	
1333	1.677	(hrcA) heat inducible transcription regulator repressor	3 STD DEV

Table 2. Mutants that displayed altered *ldh1* expression levels derived from Figure 3B

NARSA mutant	Ratio of Wildtype	Role of Regulator	
872	1.23	transcriptional regulator, AraC family	
1622	1.25	(SaeR) DNA binding response regulator	
1684	1.27	(arlR) DNA binding response regulator	
1316	1.31	(phoU) phosphate transport system regulatory protein	
386	1.38	(Rot) Staphylococcal accessory regulator	
1555	1.42	(CodY) Transcription repressor	
786	1.54	transcription regulator, MerR family	2 STD DEV
1333	1.59	(hrcA) heat inducible transcription regulator repressor	
1109	1.87	(rpoF/sigB) RNA polymerase sigma factor	
1607	1.98	(rsbU) sigma-B regulation protein	
588	2.02	(SrrB) staphylococcal respiratory response protein	3 STD DEV
1309	6.33	(SrrA) Respiratory Response Protein	
1158	34.87	(Rex) Redox Sensing Transcriptional Repressor	

Mutants of interest were selected by averaging the ratio of the wildtype for all 105 mutants and determining the standard deviation for the library. Mutants with 2 standard deviations from the mean (P=.05) and 3 standard deviations from the mean (P=.01) were determined to be significantly different from the Wildtype to justify further analysis. For *ldh1*, both Δ*srrA* and Δ*rex* were excluded from the average and standard deviation as outliers.

DISCUSSION

Mutants that had corresponding expression changes observed in both Hmp and Ldh1 suggest that the mutated regulatory protein likely affects overall transcript levels. In order to determine if the regulatory protein is a regulator for both genes, or simply affects overall transcript levels, the expression of Rpod (constitutively active) must be compared. Mutants in which all three genes show increased expression exclude these mutants from the list of regulatory proteins to investigate further. One such mutant was observed- 1555 (*CodY*). Δ*CodY* was elevated by 40% in both p*hmp::*GFP and p*ldh1*::GFP (Table 1 & 2). CodY is a known transcript repressor that would be predicted to cause increased transcription levels when mutated. Rpod expression must be analyzed in order to confirm the removal of CodY from the list of possible regulators for either gene.

Change in transcription levels of p*hmp*::GFP without a corresponding change in transcription levels of p*ldh1*::GFP and vice versa indicates a possible regulator for the gene. Of the mutants identified by altered transcription levels for p*ldh1*::GFP, *ΔsigB* and Δ*rsbU* are of particular interest. SigB is an RNA polymerase sigma factor. During transcription, sigma factors help recruit RNA polymerase to the site of transcription. *S. aureus* utilizes a normal housekeeping sigma factor in addition to multiple alternative sigma factors with specificity to different promoters. SigB is one such alternative sigma factor. The utilization of sigma factors with different specificities allows the bacteria to alter transcription in response to a changing environment. The regulation of SigB in *Bacillus subtilis* is well characterized, and *S. aureus* is suspected to have similar regulation with some key differences (Figure 4). In *S. aureus*, SigB and related Rsb proteins (W, U, and V) are conserved, and in both *S. aureus* and *B. subtilis*, RsbU conteracts inhibition of SigB by RsbW. In *S. aureus*, however, a constitutively active RsbU

appears to set a basal level of SigB activity which is subsequently increased by different cell stresses. Increased RsbU did not appear, however, to increase the ratio of RsbV/RsbV-P, indicating regulation that further differs from *B. subtilis* (Pane-Farre, 2009).

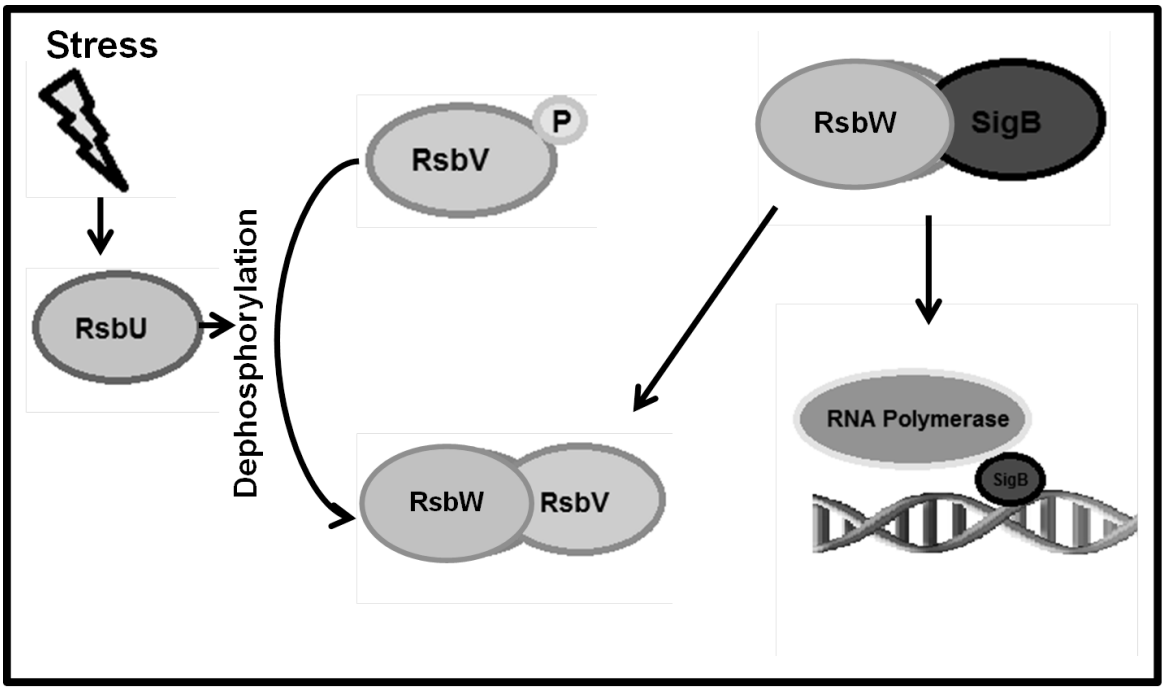

Figure 4. Regulation of SigB in *Bacillus subtilis*. In *B. subtilis* Sig B has been shown to be regulated by RsbW which forms a complex with SigB and prevents it from binding to promoter sequences. RsbV is an anti-sigma factor antagonist which competes with SigB to bind to RsbW when dephosphorylated. The release of SigB allows for binding to promoter sequences and subsequent transcription. RsbU acts as a phosphotase for RsbV during environmental stress, allowing it to form the complex with RsbW (Pane-Farre, 2009).

Similar GFP transcription in the Δ*sigB* and Δ*rsbU* backgrounds (table 2) is consistent with the conserved role of RsbU as an activator of SigB. Both *ΔsigB* and Δ*rsbU* backgrounds displayed a near two-fold increase in transcription levels. This increase is on par with that seen in Δ*srrB*, which is known to indirectly regulate ldh1 expression by increasing redox imbalance to minimize Rex activity (Richardson lab, Unpublished). This evidence linking SigB to the nitric oxide response of *S. aureus* makes it a prime candidate for further investigation. To elucidate the role of SigB in the expression of ldh1, work will be done to evaluate expression of the other Rex regulated genes in the *sigB* mutant. Additionally, the *ldh1* promoter region will be checked for

sequences specific to SigB by scanning for conserved binding sites.

Of the mutants identified by altered transcription levels for p*hmp*::GFP and p*ldh1*::GFP, Δ*hrcA* is of particular interest. HrcA is known to be a protein related to heat shock resistance in *S. aureus*, and has been shown to be significantly induced by NO• (Richardson, et. al, 2006). However, HrcA is a repressor for a subset of genes that repair heat shock damage. Included in this subset is *dnaK*. *DnaK* codes for protein refolding machinery that is critical for survival during heat stress. DnaK has also been shown to have an important role in resistance to oxidative stress which causes similar protein denaturing (Singh, 2007). The 60% increase in expression of GFP in the Δ*hrcA* mutant therefore may be due to de-repression of *hmp* and *ldh1*, or it may be due to increased stability of proteins due to the DnaK refolding machinery – resulting in persistence of GFP. To test if this phenotype is a result of action of DnaK or due to transcription regulation, the promoter regions of *hmp* and *ldh1* will be checked for binding sequence specificity to HrcA.

Future work

In order to verify the additional identified regulator candidates, Realtime PCR will be run on promising candidates from Table 1 and 2 to analyze the genomic activity with regards to *hmp* and *ldh1*. Because the screen only analyzed the activity of the reporter fusion on the plasmid, actual genomic expression can vary widely, and a small drop in promoter::GFP expression can correlate to no expression from the corresponding genomic promoter. Additionally, enzymatic activity of Ldh1 can be measured as an additional method of verification.

It is possible that the regulators will not be found in the NARSA library. This could mean that the regulator is an essential gene, and so could not be mutated. It is also possible that post

translational modification of the regulator controls the expression of *ldh1 or hmp*. These would not be found in our screen because we are not testing mutants in proteins that catalyze post translational modifications. If the regulator is not found from the screen, these other possibilities will be considered to find the regulators.

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